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Stage-related variation in rapid cold hardening as a test of the environmental predictability hypothesis

John S. Terblanche*, Elrike Marais, Steven L. Chown

Centre for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa

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Abstract

The environmental predictability (EP) hypothesis proposes that rapid cold hardening (RCH) might be common in temperate species incapable of surviving freezing events and which also dwell in unpredictable environments. The kelp fly *Paractora dreuxi* serves as a useful model organism to test this prediction at an intra-specific level because larvae and adults show different responses to low temperature despite occupying a similar unpredictable thermal environment. Here, using acclimation temperatures, which simulated seasonal temperature variation, we find little evidence for RCH in the freeze-intolerant adults but a limited RCH response in freeze-tolerant larvae. In the relatively short-lived adults, survival of −11 °C generally did not improve after 2 h pre-treatments at −4, −2, 0, 10, 20 or 25 °C either in summer- (10 °C) or winter (0 °C)-acclimated individuals. By contrast, survival of summer-acclimated larvae to −7.6 °C was significantly improved by ~37% and 30% with −2 and 0 °C pre-treatments, respectively. The finding that summer-acclimated larvae showed RCH whereas this was not the case in the winter-acclimated larvae partially supports the predictions of the EP hypothesis. However, the EP hypothesis also predicts that the adults should have demonstrated an RCH response, yet they did not do so. Rather, it seems likely that they avoid stressful environments by behavioural thermoregulation. Differences in responses among the adults and larvae are therefore to some extent predictable from differences in their feeding requirements and behaviour. These results show that further studies of RCH should take into account the way in which differences among life stages influence the interaction between phenotypic plasticity and environmental variability and predictability.

Keywords: Lethal limits; Intra-population variation; Phenotypic plasticity; Predictability; Seasonal variation; Temporal variation

1. Introduction

Rapid cold hardening (RCH) is a physiological mechanism which enables an organism to survive an otherwise lethal low temperature following pre-exposure to a less severe low temperature (Lee et al., 1987; Chen et al., 1987a). The RCH response is frequently accompanied by an immediate increase in glycerol and, on termination of the cold pre-treatment, often elicits heat shock protein production. RCH may also increase cell membrane fluidity (Lee et al., 2006a), probably through changes in poly-unsaturated fatty acid composition (Overgaard et al., 2005), which enhance cellular survival at low temperatures (Lee et al., 2006a, b). Some or all of these biochemical changes contribute to a reduction in the cellular damage typically associated with low-temperature stress (Denlinger and Lee, 1998; Lee et al., 2006b). Not only can RCH protect against non-freezing injury, but it may also increase freeze tolerance (Lee et al., 2006b). Consequently, RCH is now considered an important component of the suite of responses terrestrial invertebrates possess for coping with environmental temperature variation (Bale, 2002; Sinclair et al., 2003a, b; Lee et al., 2006b).

However, how extensive RCH is in insects, and whether any consistent environment-related pattern characterizes the expression of RCH is poorly understood (Chown and Nicolson, 2004). At present, approximately 30 terrestrial arthropod species are known to show RCH (Danks, 2005). By contrast, only a handful of species are unable to produce a cold-induced RCH response (e.g. Vandyk et al., 1996; Burks and Hagstrom, 1999; Sinclair and Chown,

*Corresponding author. Tel.: +27 21 808 2605; fax: +27 21 808 2405.
E-mail address: jst@sun.ac.za (J.S. Terblanche).

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2003; Hawes et al., 2006). Nonetheless, it has been suggested that RCH provides a mechanism enabling activity despite sudden low-temperature periods, and that it might be advantageous when temperatures are unpredictable, such as in spring and autumn (Rosales et al., 1994; Kelty and Lee, 2001).

Sinclair and Chown (2005) took this idea several steps further when they suggested, on the basis of the literature and their work on temperate African species, that RCH might be common in temperate species incapable of surviving freezing events and which also dwell in unpredictable environments. This may be termed the 'environmental predictability (EP) hypothesis'. Such environments, which seem to be especially characteristic of the temperate to high latitudes of the southern hemisphere (Vasseur and Yodzis, 2004; Chown et al., 2004), are thought to promote strategies, including moderate freezing tolerance and RCH in individuals which are not freezing tolerant, that enable insects to cope with unexpected low-temperature periods (Sinclair et al., 2003a, b; see also van der Laak, 1982; Chen et al., 1990). In particular, individuals that are not freezing tolerant are thought to show RCH because they have no other physiological means of overcoming the otherwise lethal effects of a low-temperature stress.

However, the generality of this 'environmental predictability hypothesis' remains unresolved given that investigations of RCH in insects, and particularly southern hemisphere species, are still relatively uncommon. Moreover, substantial differences in response exist between the southern hemisphere species that have been investigated. In keeping with the EP hypothesis, two beetle species incapable of tolerating freezing, *Chirodica chalcoptera* and *Afrinus* sp., show considerable RCH in response to low-temperature pre-treatments, and the freezing-tolerant caterpillars of the moth *Pringleophaga marioni* do not (Sinclair and Chown, 2003, 2006; Terblanche et al., 2005). By contrast, in the Antarctic midge, *Belgica antarctica*, summer-acclimatized larvae show RCH, while adults do not (Lee et al., 2006b). In addition, in cold-acclimated larvae freezing tolerance is improved by a low-temperature pre-treatment. Clearly, there is considerable variation in RCH responses between life stages, species and hemispheres, and there may also be some phylogenetic signal to the pattern, although insufficient evidence exists either way.

Clearly, further tests of the EP hypothesis require additional information either on (i) many species from a range of environments and encompassing the suite of responses to low temperature, (ii) species whose individuals differ substantially in their cold hardiness strategies (e.g. as a consequence of ontogeny) or (iii) on clades where species are exposed to different levels of environmental predictability (see Deere and Chown, 2006). An explicit prediction of the EP hypothesis is the absence of RCH in freezing-tolerant individuals, and its presence in individuals that lack this suite of traits either permanently or as a temporary condition, such as is found in summer-acclimatized individuals of many temperate species (Zachariassen, 1985; Leather et al., 1993). The flightless sub-Antarctic kelp fly, *Paractora dreuxi*, provides a model species with which to pursue the second approach. Individuals switch from moderate freezing tolerance in the larval stage to chill susceptibility in adulthood (Klok and Chown, 2001), despite the fact that these stages occupy very similar thermal environments. Both inhabit and feed on decomposing kelp wrack, although the adults are less tightly associated with kelp fronds than are the larvae (Crafford and Scholtz, 1987).

Therefore, in this study we use *P. dreuxi* for a within-species test of the EP hypothesis. Specifically, we determine whether RCH is present in adults but not larvae, as the EP hypothesis indicates, and whether acclimation to simulated winter and summer conditions has an influence on RCH ability (see Lee et al., 2006b). We also determine whether high-temperature pre-treatments induce a change in low-temperature survival. Several other studies have demonstrated that this can be the case and have used such assessments to gain insight into the mechanisms likely underlying RCH (Chen et al., 1991; Sinclair and Chown, 2003, 2006). Moreover, such assessments also provide information on the extent and nature of cross-tolerance, which in turn can provide significant insights into the mechanisms underlying, and the evolution of, responses to the abiotic environment (Chown and Nicolson, 2004; Pörtner, 2001).

2. Materials and methods

2.1. Study site and acclimation of animals

Paractora dreuxi (Diptera, Helcomyzidae) larvae and adults were collected using soft tweezers or aspirators, respectively, from Trypot Beach on sub-Antarctic Marion Island (46′54″°S; 37′45″°E). Larvae and adults inhabit stony beaches year-round (Crafford et al., 1986), and are capable of vertical movement through the round stony substrate (Crafford, 1984). The larval stage takes approximately 2 months, with the third instar lasting 40–50 days and being responsible for most kelp consumption. The pupal stage lasts for 30–60 days, and the adults can live for 14–21 days (Crafford, 1984) feeding on decomposing kelp slime. Adults and larvae usually avoid the intertidal zone but can be found occasionally in the splash zone (upper eulittoral zone), 3–7 m from the shoreline (Crafford and Scholtz, 1987). They are very rarely found in terrestrial vegetation, but rather keep to the stony areas of the beach.

2.2. Acclimations

The study was conducted in April 2005 during the austral autumn. Live specimens were returned to the laboratory, where they were immediately sorted for acclimations or for experimental use (usually <2 h post-capture). In the laboratory, the animals were kept in temperature-controlled cabinets at 0 or 10 °C (recorded

mean ± SD temperatures (using Thermochron DS1921 iButtons, Dallas Semiconductors, Texas): 0 ± 0.7 °C and 9.3 ± 0.5 °C) with a photoperiod set to match environmental day light cycles at this time of the year (12:12 L:D) for a period of 7 days. They were housed in 500 ml plastic jars at low density (adults: $n = 25$ per container; larvae: $n = 50$ per container) with moistened, fresh kelp. Kelp was replaced or added, moistened with fresh water, and containers were randomized between shelves on a daily basis. The 'acclimation temperatures' were chosen to represent summer and winter temperatures on Marion Island (for detailed microclimate data at sea level see Deere et al., 2006), but did not include the photoperiodic component to simplify interpretation of the effects. Seven days was selected as the acclimation period because previous work on other species both from Marion Island and elsewhere has indicated that acclimation responses equilibrate within this period (Hoffmann and Watson, 1993; Klok and Chown, 2003; Terblanche et al., 2006). A total of 200 adults and 200 larvae were exposed to each temperature.

2.3. Determination of test temperature

Although lower lethal temperatures have been determined previously using a slow cooling, ramping technique for *P. dreuxi* (Klok and Chown, 2001), we used a 'plunge' method similar to that of other RCH studies (e.g. Lee et al., 1987; Kelty and Lee, 2001; Sinclair and Chown, 2003). To determine which test temperature would induce low (but not zero) survival, we sorted field-collected adults and larvae into 60-ml polypropylene tubes in groups of 8–10 individuals. Groups were plunged into a water bath (Grant LTC 12, Grant Instruments, UK) for 2 h at the set temperatures from −2 to −14 °C and −2 to −9 °C for adults and larvae, respectively (1 °C increments). Survival, defined as co-ordinated movement and response to a stimulus, was assessed after 24 h at 10 °C. This was replicated three times per temperature group. Discriminating temperatures of −7.6 and −11.0 °C were used for larvae and adults, respectively, based on replicated logistic regression of survival curves and interpolations of the temperatures at which 25% of the sample survived (Fig. 1). Klok and Chown (2001) previously demonstrated that in field-fresh animals collected at the same time of year, adults freeze from −6.7 to −15 °C, but show some pre-freeze mortality, while larvae freeze at −3.1 to −3.9 °C.

2.4. Pre-treatment and handling controls

Larvae and adults were taken from the incubators and placed into polypropylene tubes. Tubes were placed either into a water bath (−4, −2 °C treatments) or a temperature-controlled chamber (0, 10, 20, 25 °C treatments). After 2 h, tubes were removed and placed into a water bath set to the discriminating temperatures of −7.6 or −11.0 °C for larvae and adults, respectively. After 2 h at the discriminating temperature, tubes were removed from water baths or climate chamber treatments and transferred back to their original acclimation temperatures and survival was scored after 24 h. A replicated 'handling control' was included in each acclimation group and involved plunging tubes ($n = 3$) into a water bath set to 0 or 10 °C as appropriate. The handling controls were removed after 2 h and placed at either 0 or 10 °C in temperature-controlled chambers for the 0 and 10 °C acclimations, respectively, for recovery (24 h) and subsequent scoring of survival. The order of pre-treatments was randomized during experimental days. The complete set of raw survival data is available from the corresponding author upon request.

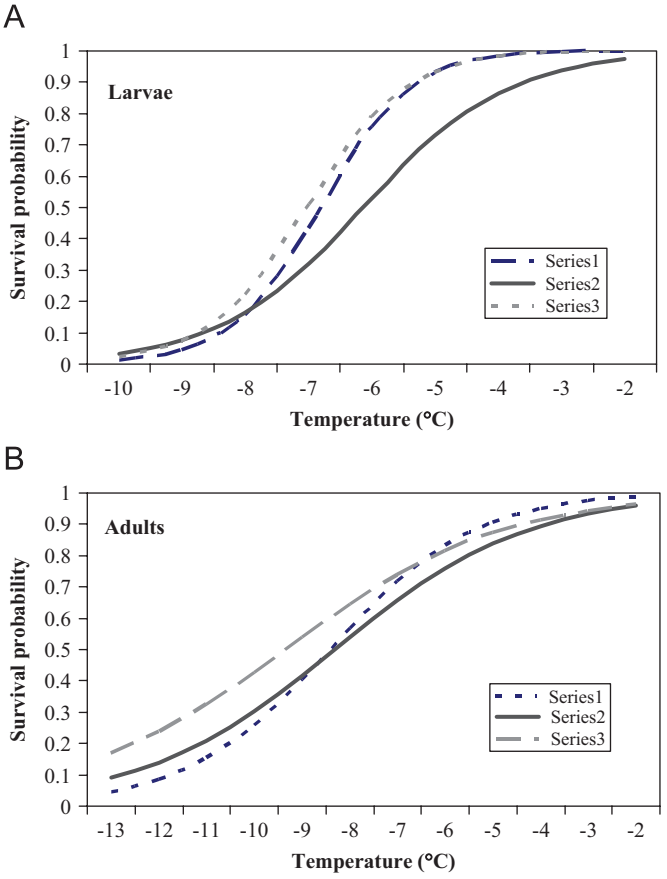

Fig. 1. Replicated lower lethal temperature curves derived from probability functions in (A) larvae and (B) adults of *Paractora dreuxi*. No pre-treatments were used during the determination of lower lethal temperatures.

2.5. Statistical analyses

Lower lethal temperature curves were obtained using PROC PROBIT in SAS (v. 9.0, SAS Institute, Cary, NC, USA) for each replicate. Initially, generalized linear model analyses were undertaken using a binomial distribution and a logit link function in Statistica 7.0 (Statsoft, Tulsa, USA) to test for effects of acclimation, pre-treatment, life-stage or interactions between these factors on survival.

Table 1
Summary of outcomes from a generalized linear model (assuming a binomial distribution, with a logit link function) investigating the effects of life stage (larvae, adults), acclimation, pre-treatment and the interactions thereof on survival in *Paractora dreuxi*

Effect	df	Log likelihood	Wald χ^2	p
Intercept	1	−442.9		
Life stage	1	−396.4	93.02	<0.00001
Acclimation	1	−395.0	2.84	0.0921
Pre-treatment	5	−389.7	10.70	0.0581
Life stage × acclimation	1	−389.4	0.66	0.419
Life stage × pre-treatment	5	−372.5	33.66	<0.00001
Acclimation × pre-treatment	5	−362.0	21.13	0.0008
Life stage × acclimation × pre-treatment	4	−355.9	12.03	0.017

Note that handling controls were not included in this analysis.

Subsequently, survival data were compared in a pair-wise fashion between each treatment and the control (handling) pre-treatment within each acclimation. Thus, in the case of 0 °C-acclimated flies and larvae, −4, −2, 10, 20, 25 °C pre-treatments were compared with a 0 °C control using pair-wise randomization analyses (Resampling Procedures v. 1.3, D. Howell, University of Vermont, USA), as frequency distributions were not normal even after transformation and assumptions of equal variances were not satisfied in some treatment groups (as in Terblanche et al., 2005). Each individual pre-treatment (−4, −2, 0, 20, 25 °C) for flies acclimated to 10 °C was compared with a handling control at 10 °C in a similar manner. A *post hoc* false discovery rate correction was used to correct for statistical artefacts of multiple tests on the null hypothesis (Garcia, 2004). Results are presented as proportion surviving ± standard error of the mean (SEM) unless otherwise stated, and significance was set at $p = 0.05$.

3. Results

Among all individuals, significant effects on survival of life stage, but not acclimation or pre-treatment were found (Table 1). In addition, significant interaction effects were detected for life stage × pre-treatment, acclimation × pre-treatment, and life stage × acclimation × pre-treatment, but not for life stage × acclimation (Table 1). In other words, the nature, direction and extent of the pre-treatment effect on survival differed among life stages and among acclimation treatments.

In larvae acclimated to 0 °C, low pre-treatment temperatures had no significant effect on survival while high pre-treatment exposures resulted in a significant and substantial decline in survivorship (Fig. 2A). Indeed, survival declined by as much as 40% following high-temperature pre-treatments. By contrast, following acclimation to 10 °C, pre-treatments had virtually the opposite effect. Here, exposure to low, but not the lowest temperatures improved survival by more than 30%, and a similar effect was found following exposure to high pre-treatment temperatures (Fig. 2A). In the adults, pre-treatments had no significant effect whatsoever (Fig. 2B). Relative to the controls, adults acclimated to 0 and to 10 °C, and pre-treated in a variety of ways, showed no significant increase or decline in survival.

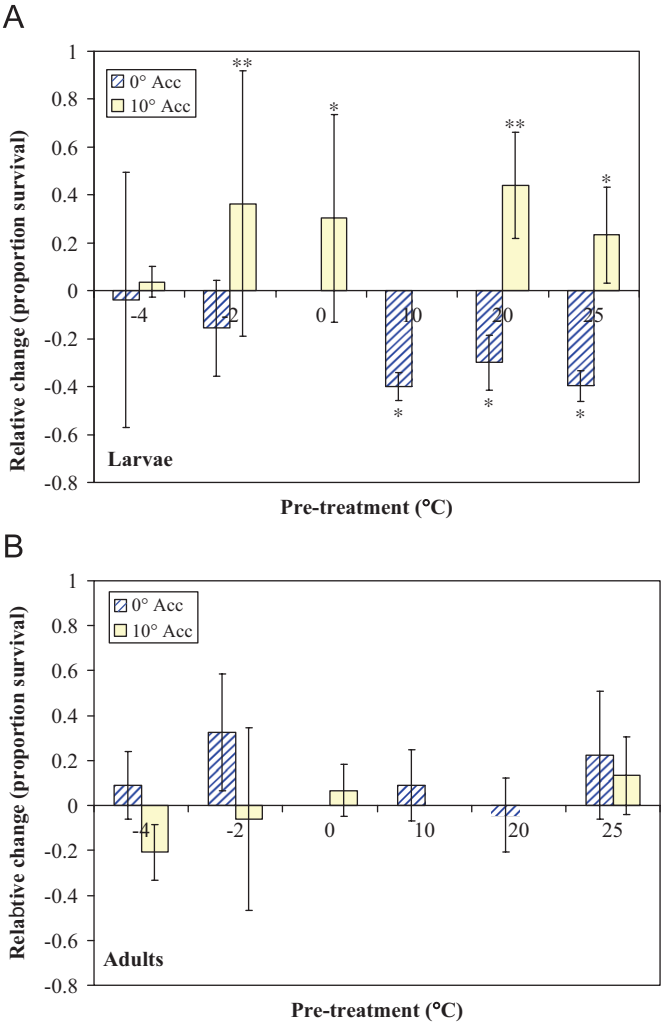

Fig. 2. Relative change in proportion survival (±SD) of *Paractora dreuxi* (A) larvae and (B) adults after temperature pre-treatments for 0 and 10 °C acclimation groups ($n = 25$–33 per treatment). Relative survival is calculated as the handling control's survival value subtracted from the experimental treatment's survival value. $^{*}p<0.05$ and $^{**}p<0.001$ after correction for multiple comparisons.

4. Discussion

The hardening responses of the larvae of *P. dreuxi* are in many ways similar to other insects. Summer-acclimated individuals show a pronounced RCH response, with survival increasing by 30–37%. Many studies have now documented such RCH (e.g. Lee et al., 1987; Chen et al., 1990; Larsen and Lee, 1994; Klok et al., 2003; Terblanche et al., 2005; Powell and Bale, 2006) although in many cases RCH may improve the survival by 50–70% (e.g. Terblanche et al., 2005; Powell and Bale, 2006; Lee et al., 2006b). Moreover, the decline in the efficacy of the pre-treatment with an increase in the stress imposed during the procedure has also been documented elsewhere (Yocum and Denlinger, 1994) and may be partly due to less individuals surviving more extreme pre-treatments, although generally the reasons for this decline are not well understood. Improved survival to a low-temperature stress following pre-treatment at a high temperature is also known from several species, including the fly *Sarcophaga crassipalpis* (Chen et al., 1991), *Pringleophaga marioni* caterpillars (Sinclair and Chown, 2003) and adults of the tenebrionid *Afrinus* sp. (Sinclair and Chown, 2006). What the reasons are for this cross-tolerance can only be surmised here, but in other species it has been shown that the upregulation of heat shock protein (hsp) synthesis is likely responsible (see Denlinger and Lee, 1998 a for review), although the role of specific hsp's is complex (Michaud and Denlinger, 2004). As in other studies (e.g. Sinclair and Chown, 2003, 2006) the role of hsp's in *P. dreuxi* requires confirmation, especially given more recent work demonstrating that the mechanisms underlying RCH may include glycerol production and alterations to fatty acid composition of membranes (Overgaard et al., 2005; Lee et al., 2006a; Yoder et al., 2006).

In the winter-acclimated larvae, the absence of a hardening response is in keeping with what might be expected of freezing-tolerant individuals (Sinclair and Chown, 2006), but contrasts strongly with the findings of Lee et al. (2006b) who documented a pronounced effect of low pre-treatment temperatures on survival of the freezing-tolerant, winter-acclimated larvae of the midge *B. antarctica*. What the ultimate reasons are for this difference are difficult to fathom, but a proximate environmental cause may be assumed: even though their winter micro-environments are buffered, *B. antarctica* larvae are still likely to encounter lower temperatures than are the larvae of *P. dreuxi* (cf. data in Lee et al., 2006b, p. 400 with data in Deere et al., 2006). More generally, this might account for the differences between the freezing-tolerant species on sub-Antarctic Marion Island (see also Sinclair and Chown, 2003) and *B. antarctica*. However, a study broader in scope, including many species from a variety of environments and which assesses phylogenetic effects, is required before any firm generalizations can be made.

By contrast, the decline in survival of winter-acclimated larvae following high-temperature treatments is not unique. found that in *Sarcophaga crassipalpis*, individuals rapidly decline in tolerance if exposed to a higher temperature (25 °C), and this seems likely to have been the case for the winter-acclimated larvae of *P. dreuxi* too. The fact that the increase in mortality was realized at a temperature as low as 10 °C suggests that the high-temperature effect was not a consequence of multiple interacting stressors, but rather was a rapid loss of the benefits of a week-long acclimation to low temperature. To date, only a few studies have examined interactions between hardening, acclimation and longer term plastic changes to low-temperature tolerance (e.g. Chen and Walker, 1994; McDonald et al., 1997, 2000; Anderson et al., 2005; Rako and Hoffmann, 2006). It is clear from these studies that the interactions are complex, and differ among species and treatments. Therefore, comprehensive understanding thereof will require considerably greater exploration of the time by intensity response space (Chown and Terblanche, 2007).

Surprisingly, the adults showed no hardening response irrespective of the acclimation or pre-treatment conditions. Although previous investigations have shown that rapid hardening effects decline with age and differ between life stages (Czajka and Lee, 1990), complete absence of hardening is unusual. Lee et al. (2006b) found similar unresponsiveness of *B. antarctica* adults and ascribed this to their short lifespan (a few days), and to their mobility which would enable them to seek out thermally buffered microhabitats (see also Jones et al., 1987). Hawes et al. (2006) found no RCH in the springtail *Hypogastrura tullbergi* and suggested that predictably mild climates (at least for the site at which they worked) were responsible for this situation. Clearly, lifespan and the scale of environmental variation need to be taken into consideration when assessing the responses of insects to environmental variability and predictability (Palumbi, 1984; Kingsolver and Huey, 1998; Chown and Terblanche, 2007).

A lifespan of a few days is unlikely to promote the development of substantial phenotypic plasticity. However, the adults of *P. dreuxi* live for 2–3 weeks, which is sufficient for them to potentially encounter a relatively wide range of temperatures (Chown and Crafford, 1992; Deere et al., 2006). Nonetheless, over their lifespans variation in temperature is likely to be highly unpredictable, presumably also selecting against pronounced plasticity (Deere and Chown, 2006). In addition, the adults are highly mobile and might also be capable of avoiding stressful conditions by simply moving to more appropriate microclimates. The stony beaches these flies occupy comprise a complex, three-dimensional habitat, and adult flies typically make use of this complexity (Crafford, 1984; Crafford and Scholtz, 1987). Therefore, rather than use physiological means to survive low-temperature stress, they might simply regulate behaviourally (see also Jones et al., 1987). In other words, behavioural regulation may preclude selection for physiological responses (Huey et al., 2003), so explaining the lack of response in the

adult flies to all of the experimentally imposed pre-treatments.

Although larvae are mobile, they depend on the kelp substrate for food, and are much slower than the adults (Crafford, 1984), so ruling out rapid behavioural responses and movement away from the kelp wrack. Moreover, because they typically dwell within a layer of slime on the kelp, which can inoculate freezing and depress survival (Klok and Chown, 2001), a rapid response (i.e. within a few hours) to low-temperature conditions is likely essential to their survival, especially in summer. On Marion Island, cold snaps in the summer months are not infrequent (Deere and Chown, 2006). In other words, for larvae of *P. dreuxi*, predictability of conditions from day to day is less relevant than prediction of conditions from hour to hour. Thus, rather than being counter-intuitive, differences among the adults and larvae (which also include a broader thermal range in larvae than in the adults (critical thermal limits: −5.1 to 35.5 °C vs. −2.7 to 30.2 °C, respectively), Klok and Chown, 2001) are to some extent predictable from their differences in feeding requirements and behaviour. Such physiological differences across the life cycle of holometabolous insects should be expected given the very different environments typically occupied by different life stages and even by different instars (see e.g. Casey et al., 1981, 1988; Gaston et al., 1991; Klok and Chown, 1999, see also Spicer and Gaston, 1999). Indeed, they are also commonly found in the thermal responses of insects (e.g. Morrissey and Baust, 1976; Chen et al., 1987b, 1991; Vernon and Vannier, 1996; Hart and Bale, 1997). However, given that adults and larvae of *P. dreuxi* occupy virtually the same habitat, it is clear that the way in which life-stage-related variation influences the interaction between phenotypic plasticity and environmental variability and predictability needs more thorough exploration.

In conclusion, the summer-acclimated larvae showed RCH whereas this was not the case in the winter-acclimated larvae, so partially supporting the predictions of the predictable environments hypothesis. However, the hypothesis also predicts that the adults should have demonstrated an RCH response, yet they did not. Clearly, the original ideas set out by Sinclair and Chown (2005) were something of an oversimplification, as both this study and that of Lee et al. (2006b) have demonstrated. Nonetheless, the environmental predictability hypothesis emphasizes the need to consider environmental stochasticity during investigations of the responses of insects to low temperatures. Further development of the hypothesis will require consideration of the traits in question in the context of the lifespan of the stage being analysed, the scale of environmental predictability, and the likely influence of behavioural responses (Kingsolver and Huey, 1998; Deere and Chown, 2006; Hawes et al., 2006; Chown and Terblanche, 2007). An explicit modelling approach such as that adopted by Voituron et al. (2002) would seem to be an excellent place to start.

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